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MOLECULAR PATHOGENESIS

Persistent *Toxoplasma* Infection of the Brain Induced Neurodegeneration Associated with Activation of Complement and Microglia

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ABSTRACT *Toxoplasma gondii*, a common neurotropic parasite, is increasingly being linked to neuropsychiatric disorders, including schizophrenia, Alzheimer's disease, and Parkinson's disease. However, the pathogenic mechanisms underlying these associations are not clear. *Toxoplasma* can reside in the brain for extensive periods in the form of tissue cysts, and this process requires a continuous immune response to prevent the parasite's reactivation. Because neuroinflammation may promote the onset and progression of neurodegenerative diseases, we investigated neurodegeneration-associated pathological changes in a mouse model of chronic *Toxoplasma* infection. Under conditions of high-grade chronic infection, we documented the presence of neurodegeneration in specific regions of the prefrontal cortex, namely, the anterior cingulate cortex (ACC) and somatomotor cortex (SC). Neurodegeneration occurred in both glutamatergic and GABAergic neurons. Neurons that showed signs of degeneration expressed high levels of CX3CL1, were marked by profoundly upregulated complement proteins (e.g., C1q and C3), and were surrounded by activated microglia. Our findings suggest that chronic *Toxoplasma* infection leads to cortical neurodegeneration and results in CX3CL1, complement, and microglial interactions, which are known to mediate the phagocytic clearance of degenerating neurons. Our study provides a mechanistic explanation for the link between *Toxoplasma* infection and psychiatric disorders.

KEYWORDS CX3CL1, neurodegeneration, neuroinflammation, *Toxoplasma gondii*, complement, microglia

It is increasingly evident that the brain is not an immune-privileged site, and inflammatory events occur during central nervous system (CNS) injury, exposure to toxic metabolites, infection, autoimmunity, and aging (1). This neuroinflammation acts as a protective mechanism for controlling neural insults. However, this protective response is often accompanied by diverse brain pathologies, such as synaptic loss, lesion, and neuronal cell death (2). Therefore, activation of the immune system within the CNS is widely found in psychiatric disorders, such as schizophrenia, and in neurodegenerative diseases, such as Alzheimer's and Parkinson's disease (3, 4). For instance, activation of microglia and the complement system are major components of inflammation in neurodegenerative diseases. Microglia have been postulated to clear degenerating neurons that are tagged by complement proteins via the fractalkine (CX3CL1) pathway (3, 5, 6).

Toxoplasma gondii is an intracellular parasite that infects approximately a billion people worldwide. Although initial infection with *Toxoplasma* is associated with few symptoms in immunocompetent individuals, the infection often becomes chronic and persists lifelong in the form of tissue cysts in the brain, retina, and muscles (7). Little is known about the effects of lifelong brain infections on the neurological health of

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people. Recent discoveries suggest that exposure to *Toxoplasma* increases the risk for neuropsychiatric disorders, such as schizophrenia (8), Alzheimer's disease (9), and Parkinson's disease (10). In the elderly, *Toxoplasma* seropositivity has been associated with alterations in cognitive functioning and overall cognitive decline (11). In animal studies, *Toxoplasma* infection has been reported to induce neophobia and to have behavioral effects on learning and memory, anxiety, and locomotion (12). Several studies have investigated neuropathological changes in *Toxoplasma*-infected brain. In mice with chronic toxoplasmosis, impaired fiber density and fiber continuity were revealed by analysis of neuronal connectivity in infected regions, particularly within somatosensory areas (13). Another study reported alterations in neuronal morphology (e.g., a reduction in dendritic spines) and network activity in brains of *Toxoplasma*-infected mice (14). Several lines of evidence support a causal role for *Toxoplasma* infection in disruption of glutamate signaling (14–16). In human studies, *Toxoplasma*-positive schizophrenic patients exhibited a significant reduction of gray matter volumein cortical regions compared with that of *Toxoplasma*-negative patients (17).

The mechanism by which *Toxoplasma* infection leads to neurodegenerative diseases is not clear. Neuroinflammation is postulated to be crucial in the onset and progression of neurodegenerative diseases (18). Studies show that *Toxoplasma* cysts can be found almost exclusively in neurons (19, 20) and are controlled mainly by cell-mediated immunity provided by resident CNS and infiltrating peripheral immune cells (21). Moreover, a low-grade brain inflammation has been documented in rodent models of chronic *Toxoplasma* infection characterized by microglia and astrocyte activation (22, 23), an increase in complement protein C1q (22, 24), and ventricular dilatation (22). These findings raise concerns about the possibility of neurodegeneration in *Toxoplasma*-infected brain, as neuroinflammatory responses might persist for years if infection is not resolved. In this study, we examined brains of *Toxoplasma*-infected mice for signs of neurodegeneration using Fluoro-Jade B (FJB), a known marker for degenerating neurons (25). We show that persistent *Toxoplasma* infection leads to cortical neurodegeneration affecting both glutamatergic and GABAergic neurons. Neurons that show signs of degeneration are characterized by complement deposition and overexpression of CX3CL1, a chemokine that binds to the CX3CR1 receptor on microglia (26). Activated microglia were found to accumulate at the site of degeneration, embracing and surrounding degenerative neurons. Our study identifies neurodegenerative effects as a possible mechanistic basis for the association of chronic *Toxoplasma* infection with neuropsychiatric diseases.

RESULTS

Cyst-associated neurodegeneration presents in the SC and ACC of mouse brain. We infected outbred CD-1 mice with a virulent type I strain of *Toxoplasma*. Mice were stratified into five groups based on antibody profiles, as follows: (i) an unexposed control group, (ii) an IgG-positive and highly matrix antigen 1 (MAG1)-positive (IgG+/MAG1+ high) group (with a high-grade infection and a MAG1 level of $\geq$0.5), (iii) an IgG+/MAG1+ low group (with a low-grade infection and a MAG1 level of $<$0.5), (iv) a *Toxoplasma*-exposed group that did not develop MAG1 antibody (IgG+/MAG1−), and (v) a *Toxoplasma*-exposed group that did not develop an antibody response (IgG−/MAG1−). *Toxoplasma* IgG is an indicator of exposure to the parasite, while MAG1 antibody serves as a serologic marker for cyst burden (27). We conducted staining with FJB, a well-known marker for neurons that undergo degeneration, of brain sections of the mice. Our characterization of neurodegeneration focused on the cortex and the striatum region based on studies implicating these regions in neurodegenerative diseases broadly (28, 29). Among the five groups, strong cortical FJB staining was found only in mice with high MAG1 antibody levels (Fig. 1). There were no FJB-positive cells in mice from the other groups (see Fig. S1 in the supplemental material). In MAG1+ high mice, cells positive for FJB were predominantly located in the somatomotor cortex (SC) and anterior cingulate cortex (ACC) (Fig. 1), while FJB-positive cells were scarce in the other regions of cortex. Higher magnification showed that FJB stained the cell

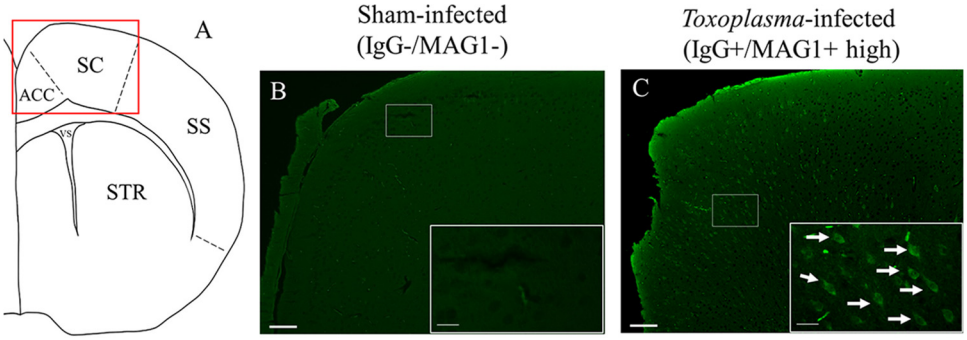

FIG 1 Brain examination revealed neurodegeneration in the anterior cingulate cortex (ACC) and somatomotor cortex (SC) of mice with high-grade chronic *Toxoplasma* infection. (A) Anatomical diagram illustrating the ACC and SC. STR, striatum; SS, somatosensory system; VS, ventricular system. Coronal sections of *Toxoplasma*- and sham-infected mice at 5 months postinfection are shown. FJB staining was performed to evaluate neuronal degeneration. (B) No FJB-positive cells were observed in the ACC and SC of the sham-infected mice. (C) Numerous FJB-positive cells were visualized in the ACC and SC of the MAG1 high mice (arrows in the inset white box at ×40 magnification). The presence of IgG is indicative of exposure to *Toxoplasma*, while MAG1 antibody serves as a serologic marker for cyst burden. MAG1 high, optical density of >0.5. Scale bar, 100 μm or 20 μm (insets). Images are representative of staining for 3 mice/per group.

bodies and processes of the cells (Fig. 1C, inset). We also performed FJB staining in combination with staining for the neuronal marker NeuN. Double-fluorescence staining showed the expected overlap between FJB and NeuN (Fig. 2, upper panels), confirming that the degenerating cells were neurons. To see if degenerating cells are associated with parasite antigens, we performed double staining with *Toxoplasma* antigen and FJB. However, there was no parasite-positive staining in degenerating cells (Fig. S2).

Both glutamatergic and GABAergic neurons display degeneration. There are two dominant families of neurons in the cortex, excitatory glutamatergic neurons and

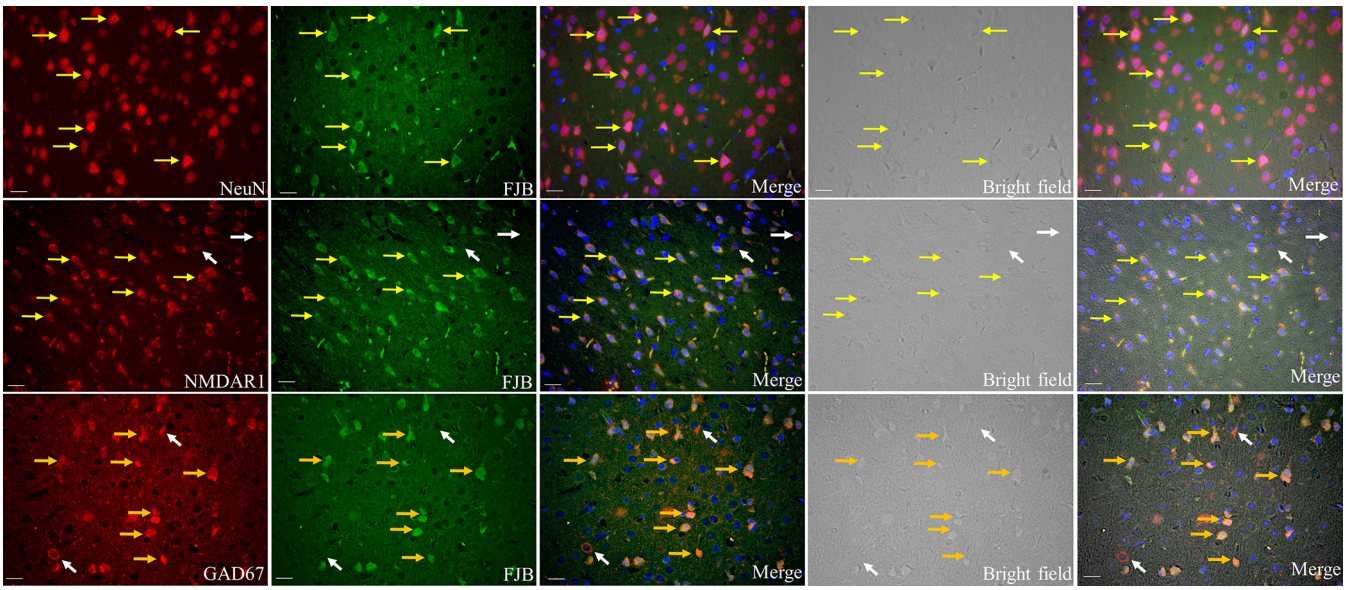

FIG 2 The FJB-positive degenerating cells were glutamatergic and GABAergic neurons. Coronal sections of MAG1 high mice were subjected to dual immunofluorescence staining of FJB with NeuN, NMDAR1, and GAD67 at the anterior cingulate and somatomotor cortex. DAPI was used in order to identify nuclei (blue). Images of cells were obtained by bright-field light (fourth column). To visualize better the NMDAR1, GAD67, and FJB staining colocalized into the cell, we superimposed the bright-field images upon merged images of fluorescence (fifth column). (Upper panels) Double staining of NeuN (red) and FJB (green) showed that all FJB-positive cells were colocalized with NeuN (yellow fluorescence, arrows), confirming that the degenerating cells were neurons. (Middle panels) Double staining of NMDAR1 (red) and FJB (green) showed that most NMDAR1-stained glutamatergic neurons were colocalized with FJB (yellow arrows), though some were not (white arrows). (Lower panels) Double-labeling of GAD67 (red) and FJB (green) showed that most GAD67-stained GABAergic neurons were colocalized with FJB (yellow arrows), though some were not (white arrows). Scale bar = 20 μm for all panels. Images are representative of staining from 3 animals.

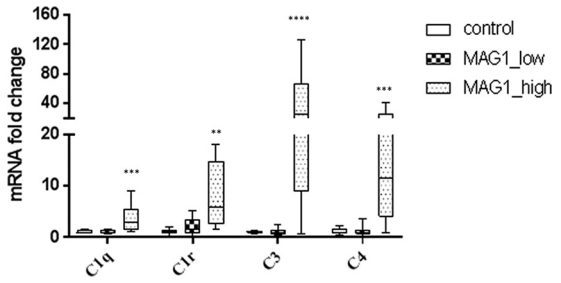

FIG 3 Cyst-associated upregulation of cortical complement components. Outbred CD-1 mice were infected with a virulent type I strain of *Toxoplasma* for 5 months. mRNA levels of C1q, C1r, C3, and C4 were elevated in brains of MAG1 high mice compared to levels in MAG1 low and control mice (8 mice per group). The box and whisker plot represents the $\Delta\Delta C_T$ distribution, the bottom and top of the box are the first and third quartiles, and the band inside the box is the median. The ends of the whiskers represent the minimum and maximum values of the data. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

inhibitory GABAergic neurons. To ascertain whether *Toxoplasma*-induced neurodegeneration is cell type specific, neurons were double-fluorescence FJB stained with markers of glutamatergic or GABAergic neurons. Fluorescence immunohistochemistry was carried out for NMDA receptor 1 (NMDAR1), a marker of glutamatergic neurons, and glutamic acid decarboxylase 67 (GAD67), a marker of GABAergic neurons, as described previously (30, 31). Images of cells were obtained by bright-field light. In brains of MAG1 high mice, most NMDAR1-stained glutamatergic neurons were colocalized with FJB (Fig. 2, middle panels, yellow arrows), though some were not (white arrows). Similarly, FJB staining is present in many but not all GAD67-stained glutamatergic neurons (Fig. 2, lower panels). These results suggest that chronic *Toxoplasma* infection causes degeneration of both glutamatergic and GABAergic neurons.

Cyst-associated elevation of cerebral complement components. Recent evidence demonstrates that the classical complement system is active in various neurodegenerative and age-related diseases (3). Here, we evaluated whether the complement cascade is upregulated in brains when parasite-driven neurodegeneration was present. We measured levels of several complement components in the prefrontal cortex from *Toxoplasma*- and sham-infected mice, including initiators of the classical pathway C1q and C1r, the central component C3, which also initiates the alternative pathway, and the downstream complement component C4.

The mRNA levels of these complement components were substantially and significantly elevated in MAG1 high mice relative to levels in MAG1 low or control mice, as revealed by real-time reverse transcription-PCR (RT-PCR). As depicted in Fig. 3, mRNA expression was increased 3.8-fold for C1q, 7.8-fold for C1r, 40.2-fold for C3, and 15.9-fold for C4 in mice with high levels of MAG1 antibodies compared to those of controls (8 mice/group). We next measured complement proteins in homogenates of prefrontal cortex to confirm the increases found at the RNA level. In mouse brain, C1q antibodies specifically detected a band at 30 to ~40 kDa (Fig. 4A, corresponding to the C1q A chain), C1r antibodies detected a band at 60 to ~80 kDa (Fig. 4B), and C4 antibodies detected a band at 80 to ~110 kDa (Fig. 4D). Using a monoclonal anti-C3 antibody, we detected the α-chain (115 kDa), the β-chain (70 kDa), and C3c alpha chain fragment 2 (43 kDa) (Fig. 4C). We found that these proteins were profoundly upregulated in MAG1 high mice, as evidenced by the 34-fold increase for C1qA, the 2.7- to ~4.5-fold increase for C3 subtypes, and an increase from undetectable to detectable levels for C1r and C4, in a comparison with controls (4 mice/group). These protein results are highly consistent with the mRNA data.

Complement C1q and C3 deposited on the surfaces of FJB-positive cells. Complement proteins C1q and C3 appear to be particularly important in tagging weak or unnecessary synapses for removal via microglial complement receptor-mediated phagocytosis (32). Given the activation of the complement cascade based on our RNA and protein studies, we next visualized the extent to which these complement com-

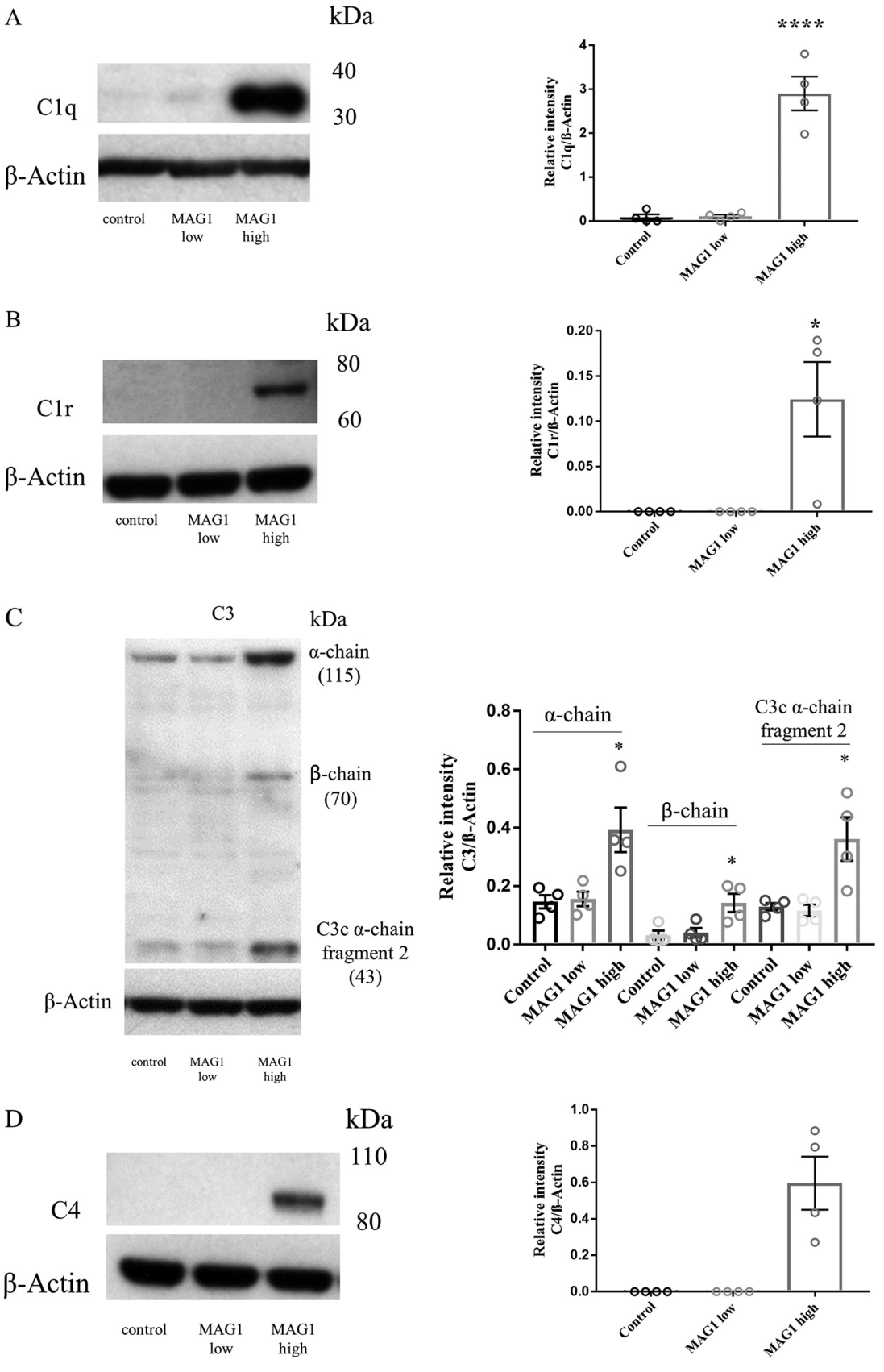

FIG 4 Cyst-associated elevation of cortical complement proteins. Outbred CD-1 mice were infected with a virulent type I strain of *Toxoplasma* for 5 months, and Western blot analysis of complement proteins C1q (A), C1r (B), C3 (C), and C4 (D) in cortical

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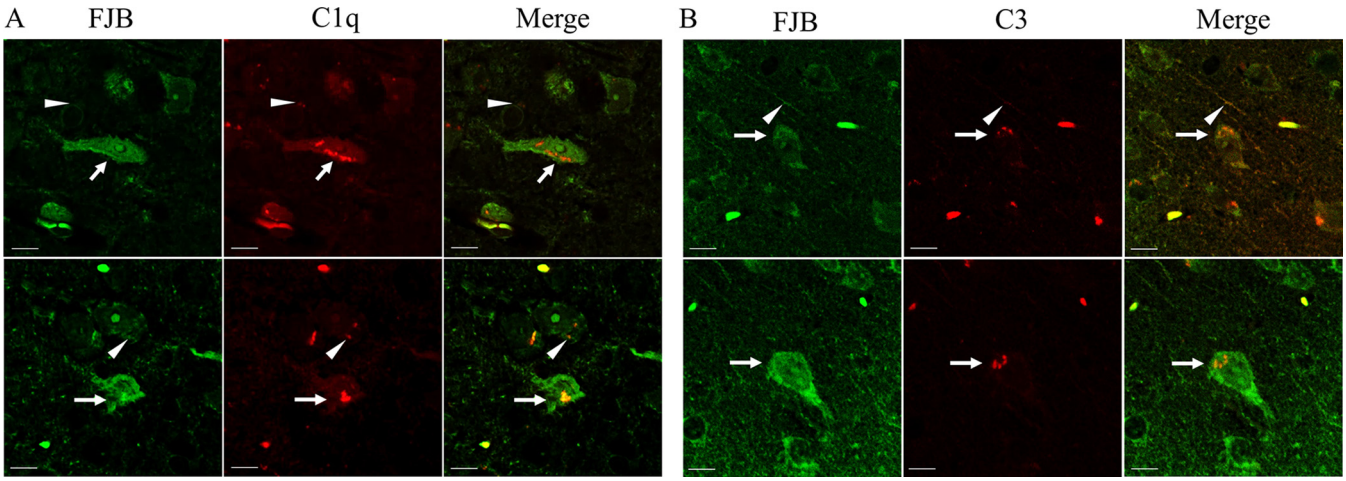

FIG 5 Confocal microscopy showed that complement proteins deposited on the surfaces of degenerative neurons. Coronal sections of MAG1 high mice were subjected to dual-immunofluorescence staining of FJB with complements C1q (A) and C3 (B). The staining of C1q (red) and C3 (red) was associated with FJB-positive (green) cells; deposition was detected on the cell bodies (arrows) as well as along cell processes (arrowhead). Scale bar = 10 μm for all panels.

ponents were deposited on degenerating neurons. Sections containing the FJB-positive cells were immunostained for C1q and C3 and analyzed by confocal microscopy. As shown in Fig. 5, we found that C1q and C3 staining were associated with FJB-positive cells, whereas deposition of C1q and C3 were detected on the surfaces of cell bodies (arrows) as well as along cell processes (arrowhead).

Degenerating neurons were surrounded and infiltrated by activated microglia. Microglia are resident phagocytes in the brain that sense pathological tissue alterations, such as synapse loss and lesion. They have been recognized as key players in neurodegenerative lesions (4). To determine whether microglia are associated with cortical neurodegeneration, we performed FJB staining in combination with a marker of microglia, IBA1, and sections were imaged by confocal microscopy (Fig. 6D, E, and F represent Z-stacks of 3 confocal scans, with a total thickness of 5 μm). Microglial activation can be identified by changes in shape and density. In sham-infected animals, the prefrontal cortex contained a few resident microglia displaying small perikarya that projected thin processes (Fig. 6A). In contrast, the level of IBA1-labeled microglia at the site of neurodegeneration increased markedly in MAG1 high mice (Fig. 6D). Moreover, IBA1-positive cells displayed activated cell morphology showing enlarged perikarya and thicker processes (Fig. 6D). IBA1 and FJB coimmunostaining showed an intimate interrelationship between microglia and neurodegenerating cells. As shown in Fig. 6F, IBA1-positive microglia can be seen completely engulfing FJB-positive cells (arrows).

Fractalkine (CX3CL1) increased significantly in degenerating neurons. It has been proposed that fractalkine (CX3CL1) signaling mediates the communication between neurons and microglia (26), because CX3CL1 is produced by neurons and its sole receptor is expressed on microglia. Given the elevated number of activated microglia at the site of neurodegeneration, we wondered whether CX3CL1 would be upregulated by degenerating neurons. In MAG1 high mice, cellular CX3CL1 was highly expressed compared to expression levels in sham-infected mice (Fig. S3). Neurons that positively stained with CX3CL1 colocalized with neurons that positively stained with FJB (Fig. 7), suggesting that neurons that undergo degeneration express high levels of CX3CL1.

DISCUSSION

Synapse loss and neuroinflammation characterize many neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis (3, 4).

FIG 4 Legend (Continued)
extracts from MAG1 high, MAG1 low, and control mice, alongside β-actin loading controls, was performed. Histograms indicate densitometric analysis of blots, expressed as means ± SEM. Analysis was performed in three independent experiments. The circles show individual values from one representative experiment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

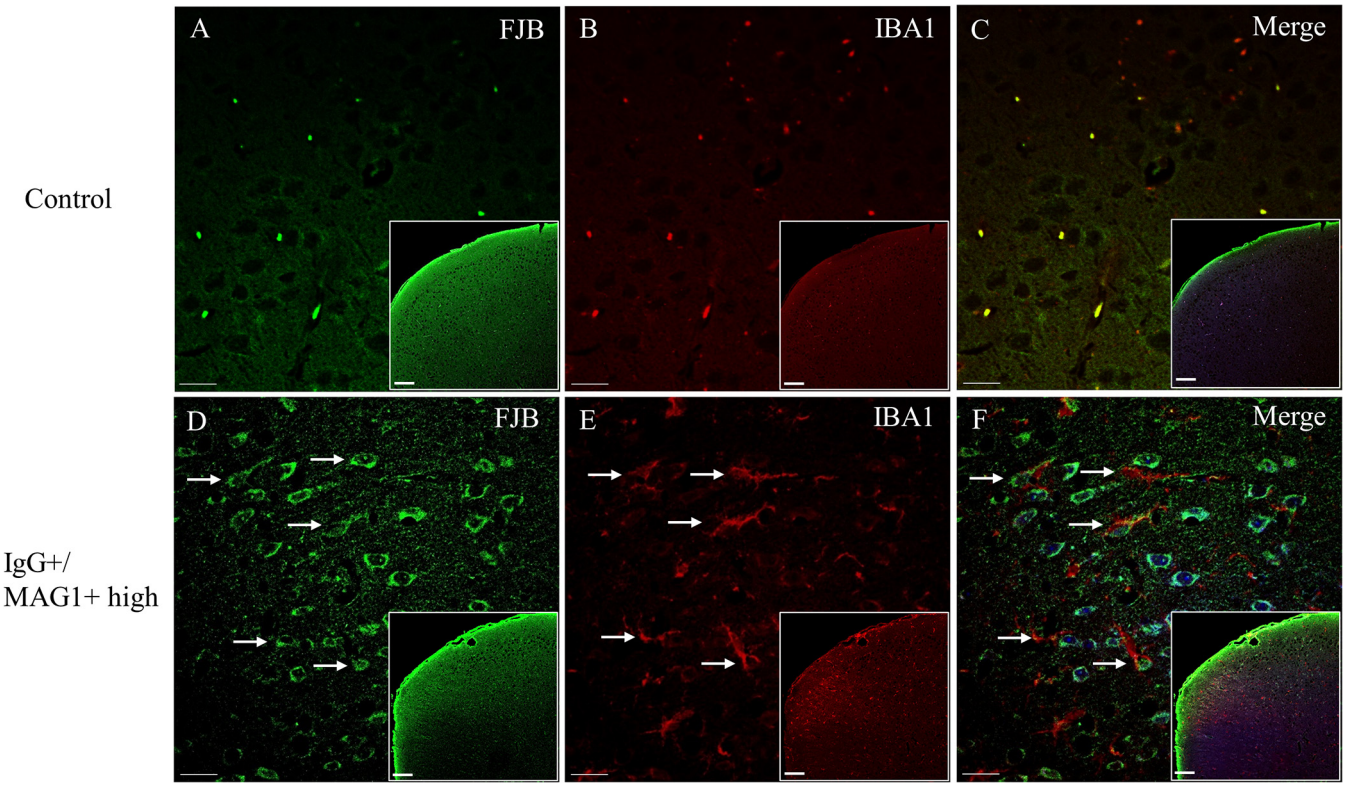

FIG 6 Confocal microscopy showed activated microglia surrounding degenerate neurons. Coronal sections of MAG1 high and control mice were subjected to dual-immunofluorescence staining of FJB (green) with IBA1 (red) at the anterior cingulate and somatomotor cortex. An increased microglial population density with activated cell morphology in MAG1 high mice was observed (E, arrows) compared to that of uninfected controls (B). The activated microglial cells were in close vicinity of degenerating neurons, embracing and engulfing them (F, arrows). Scale bar =100 μm (inset) or 20 μm for all panels.

Toxoplasma has been implicated in several neurodegenerative diseases (33), but the mechanisms underlying such associations are not known. We set out to test the effect of chronic *Toxoplasma* infection on neuronal degeneration in mice and to explore the potential mechanisms that are involved. We show that high-grade chronic *Toxoplasma* infection caused neurodegeneration in specific regions of the prefrontal cortex, namely, the anterior cingulate cortex (ACC) and somatomotor cortex (SC). Both glutamatergic and GABAergic neurons were affected, indicating nonspecific neuropathology. Neurons that showed signs of degeneration expressed high levels of CX3CL1 and were tagged by complement proteins; both processes are known to recruit and activate nearby microglia. Indeed, activated microglia were amply present at sites of neurodegeneration and closely interacted with degenerate neurons. Our experimental mouse model of *Toxoplasma* infection simulated many aspects of the initiation and pathogenesis of neurodegenerative diseases and may provide a mechanistic explanation for the involvement of chronic *Toxoplasma* infection in neurodegenerative diseases.

Neurodegeneration, identified by FJB-labeled neurons, was present in MAG1 high mice but not in MAG1 low or MAG1-seronegative mice. This result suggests that parasite burden is a major determinant for neuronal degeneration, as we have previously shown that the MAG1 antibody response is a reliable measure of cyst burden (27). However, parasite antigens were not found in degenerating cells. Therefore, it is likely that neurodegeneration is an indirect effect of the parasite infection. Several studies found that pathogen burden was positively correlated with the degree of neuroinflammation. In a study reported by Evans et al. (23), activated microglia were found in all *Toxoplasma*-infected rats with cysts but not in *Toxoplasma*-infected rats without cysts. Using a murine model of human African trypanosomiasis, Rodgers et al. (34) found a concomitant increase in the degree of neuroinflammation as the infection progressed in the brain. Given that neuroinflammation has been implicated in the etiology of

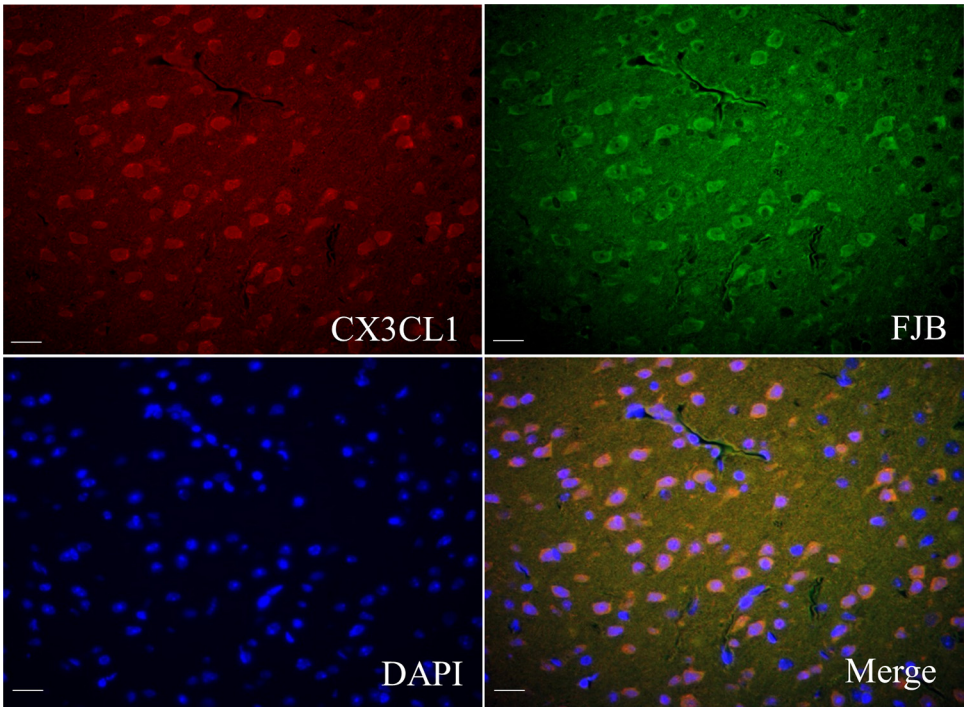

FIG 7 CX3CL1 was upregulated and localized to degenerating neurons. Coronal sections of MAG1 high mice were subjected to dual-immunofluorescence staining of FJB (green) with CX3CL1 (red). Representative microscopy images showed that CX3CL1-positive cells were colocalized to the FJB-positive cells (merge), suggesting that neurons that are undergoing degeneration express high levels of CX3CL1. DAPI staining of nuclei (blue). Scale bar = 20 μm for all panels.

neurodegenerative diseases (18), the brain inflammation generated by the presence of cysts might be a critical factor driving neural degeneration. We did not detect neurodegeneration in mice with low-grade infection (MAG1 low), presumably due to an insufficient level of cyst-derived antigens; these antigens are needed to stimulate a robust inflammatory response. Our finding that different *Toxoplasma* serological profiles are associated with the presence or absence of neurodegenerative pathology may be important in interpreting the conflicting epidemiological studies reporting associations of *Toxoplasma* infection and neurodegenerative disorders (9, 11, 35, 36). In these studies, chronic *Toxoplasma* infection is often confirmed only by seropositivity to the whole organism, and the presence or burden of the organism is not determined.

Inflammatory processes that effectively control parasite reactivation are potential candidates triggering neurodegeneration; however, the molecular cascades leading to neurodegeneration are unclear. Evidence of an inflammatory response in *Toxoplasma*-infected brain includes activation of microglia and astrocyte, an increase in cytokines and chemokines (e.g., CXCL9, CXCL10, and CCL5), and recruitment of immune cells (22, 23, 37). Given that the infection lasted a long period of time (5 months postinfection) and has a chronic progressive course, we do not have data for the evolution of the CNS inflammatory profile. Therefore, inflammatory factors that initiate and cause the neurodegenerative processes to progress are difficult to identify. Indeed, how the inflammatory response affects neuronal populations and contributes to neurodegenerative disease in general remains a critical and unanswered question (38). We showed here that, after the neuron degenerated, activated microglia engaged in phagocytosis by engulfment of synapses, likely via a complement-dependent mechanism, as well as in response to receptor-mediated recognition of specific molecular tags at the synapses.

Degenerating neurons were prominently present in specific regions of the prefrontal cortex, including in the ACC and SC, two areas that are also prominently associated with cognitive function (39, 40). The reason for selective involvement of these regions is

unclear. Although tissue cysts are randomly distributed throughout the brain, several studies have found that inflammation and parasite burden are greater in the cortex than in other areas of the brain (22, 23), which may provide an explanation for the particular targeting. In Alzheimer's disease, neurons of the hippocampus and entorhinal cortex are the first to degenerate, whereas in Parkinson's disease, dopaminergic neurons in the substantia nigra degenerate (18). Therefore, it is commonly recognized that different diseases affect different neuronal populations, though the mechanisms for their selective targeting remain poorly understood. It is of note that neurodegenerative diseases can progress over time, involving one brain region followed by another and then another (41). It is possible that *Toxoplasma*-induced degenerative pathology over time may involve other areas of the brain.

There are two dominant families of neurons in the cortex, GABAergic and glutamatergic neurons, which establish inhibitory and excitatory synapses, respectively. We found that both types of neurons exhibited degeneration. Previously, GABAergic and glutamatergic synapses and signaling have been reported to be altered upon *Toxoplasma* infection (14–16, 42). Neurons that show signs of degeneration can potentially lead to changes in neuronal connectivity and synaptic plasticity. As observed by previous studies, alterations of GABAergic signaling have been linked to seizures, Alzheimer's disease, temporal lobe epilepsy, and schizophrenia (14). Altered glutamate signaling are linked to many neurological conditions, such as schizophrenia, Parkinson's disease, cognition, addiction, anxiety, and depression (43). With respect to the pathology of neurodegenerative diseases, our findings support the involvement of *Toxoplasma* in these diseases.

We previously reported that levels of C1q are increased in the brains of MAG1 high mice and can be found as punctate patterns, which are indicative of synapses along neuronal cell processes (24). The present study extends these previous findings by showing that additional complement components (C1r/C3/C4) are activated and deposited on degenerative neurons. We demonstrated, at both the RNA and protein levels, a dramatic upregulation of components of the complement cascade in MAG1 high mice. Although the blood brain barrier normally protects the brain from plasma-derived complement, brain-resident neurons and glial cells, most often in response to injury or inflammatory signals, can locally synthesize many complement components (44, 45). We found that C1q and the downstream complement protein, C3, target degenerating neurons. Given complement's well-ascribed role in synaptic pruning (3), the function of these immune molecules appears to be to mark dysfunctional neurons for removal.

There is significant microglia recruitment and activation at the site of neurodegeneration, possibly recruited by increased levels of the chemokine CX3CL1. As described before, CX3CL1 binds to the CX3CR1 receptor on microglia, a crucial receptor regulating microglial activation in the CNS and cellular communication between microglia and neurons (26). Microglial activation may also be triggered by upregulated complement C3, as C3 receptors are expressed on microglia (46). This binding promotes phagocytosis. Accumulating evidence suggests that microglia have a well-ascribed role in clearing out dying cells in the aged and diseased brain (47). Here, we found that microglial branches surrounded the degenerating neurons in an intimate manner, the purpose of which may be to remove degenerate neurons that are tagged by complement proteins and to control inflammatory processes. These results are in accordance with recent literature where complement-mediated phagocytosis is involved in the pathology of the ageing brain and neurodegenerative diseases (3, 4).

Our study suggests that neurodegenerative processes are involved in the pathogenesis of murine toxoplasmosis and may contribute to changes in neuronal connectivity, synaptic plasticity, and behavior. This mouse model raises questions of whether *Toxoplasma* can cause or contribute to degenerative pathology in the brains of humans. In our model, fractalkine was highly expressed by the degenerative neurons and played a role in microglial recruitment. However, whether this translates to humans is unclear, because the functions of the fractalkine/CX3CR1 axis differ in humans and

mice (48). The link between *Toxoplasma* and mechanisms associated with neurodegenerative diseases is potentially important because this parasite infects approximately a billion people worldwide. Our result also suggests that decreasing antigen availability, through gradual resolution of infections or intervention strategies that promote parasite control, might help to alleviate or cure symptoms of neurodegeneration. In a recent study (49), we showed that immune checkpoint blockade directed against the programmed death 1 (PD-1) pathway results in a significant reduction in brain cyst number (77% lower), which offers a novel method to treat chronic toxoplasmosis. Another study reported increased expression of PD-L1 in brain tissue of *Toxoplasma*-infected mice (22).

MATERIALS AND METHODS

Chronic *Toxoplasma* type I infection. All mouse specimens were collected as part of a previously published study (27); no additional animals were sacrificed for the present study. As described before (27), 7- to 9-week-old female outbred CD-1 mice (ICR-Harlan Sprague) were infected intraperitoneally (i.p.) with 500 *Toxoplasma* GT1 strain tachyzoites (type I, virulent). Control mice received vehicle only (phosphate-buffered saline [PBS]). The type I strain was chosen for study in light of our previous epidemiological studies, where we found that virulent strains, such as those of type I, are associated with increased frequency and severity of human toxoplasmosis (50–52). To establish a chronic infection with this virulent strain, both control and infected mice were treated with anti-*Toxoplasma* chemotherapy (sulfadiazine sodium) in drinking water (400 mg/liter; Sigma) from days 5 to 30. Mice were sacrificed at 5 months postinfection. Brains were harvested and divided sagittally. Half of the brain was used for immunofluorescence staining, and the other half was used for dissection of the prefrontal cortex for mRNA and protein analysis.

This model generates varied outcomes ranging from aborted to severe infections, i.e., various degrees of cyst burden (27). Exposure to *Toxoplasma* was confirmed using serological measurement of anti-*Toxoplasma* antibodies with enzyme-linked immunosorbent assays (ELISAs) (IBL America). Cyst burden was measured with matrix antigen 1 (MAG1) antibody by using previously developed cyst MAG1 assays (27, 53). According to antibody profiles, mice were stratified into five groups: (i) non-*Toxoplasma*-exposed control mice (IgG−/MAG1−), (ii) exposed mice with a high MAG1 antibody level (IgG+/MAG1+ high [MAG1 level of ≥0.5]); (iii) exposed mice with a low MAG1 antibody level (IgG+/MAG1+ low [with a MAG1 level of <0.5]), (iv) exposed mice that did not develop MAG1 antibody (IgG+/MAG1−), and (v) exposed mice that did not develop an antibody response (IgG−/MAG1−).

Fluoro-Jade B staining. Fluoro-Jade B (FJB) is a fluorescent dye that specifically labels degenerating neurons (25). To perform FJB staining, paraffin-embedded specimens were cut into 5-μm sections. After deparaffinization and rehydration, the sections were immersed in a solution containing 0.06% potassium permanganate for 10 min with gentle shaking. Brain sections were then placed in a 0.0004% FJB solution for 20 min. After being washed in distilled water, the sections were mounted with prolong diamond antifade mountant (ThermoFisher Scientific).

Immunofluorescence staining. Immunohistochemistry for all antibodies was performed using standard protocols. Paraffin wax-embedded sections were cut coronally from the hemisphere at a thickness of 5 μm. Serial sections (3 mice per group; 5 sections per mouse) were treated in xylene and a graduated alcohol series and rinsed in distilled water. Antigen retrieval was performed by boiling the sections in antigen unmasking solution (Vector Laboratories; item H-3300) for 30 min. Sections were first blocked with 10% normal donkey serum for 30 min and then with 0.1 mg/ml Fab fragment donkey anti-mouse IgG (Jackson ImmunoResearch; item 715-007-003) for 1 h. Sections were stained overnight using the following primary antibodies: anti-IBA1 antibody (polyclonal, item 019-19741, 1:200; Wako), anti-C1q antibody (monoclonal, item ab71089, 1:100; Abcam), anti-C3 antibody (monoclonal, item ab225539, 1:2,000; Abcam), anti-CX3CL1 antibody (monoclonal, item ab25088, 1:500; Abcam), anti-GLUN2B antibody (monoclonal, item ab93610, 1:250; Abcam), anti-NeuN antibody (monoclonal, item ab104224, 1:1,000; Abcam), anti-GAD67 (monoclonal, item ab213508; Abcam), and anti-*Toxoplasma* (polyclonal, item PU125-5UP, 1:100; BioGenex). Double-fluorescence staining was performed for these markers in different combinations.

Secondary antibodies were purchased from Life Technologies, Inc. Finally, the sections were stained with DAPI (4′,6-diamidino-2-phenylindole; Sigma-Aldrich) and mounted with prolong diamond antifade mountant (ThermoFisher Scientific). Images were visualized using an Olympus BX41 microscope and a reflected fluorescence system. A confocal laser microscope (Zeiss LSM700) was used to verify the colocalization or intimate associations.

Immunoblot analyses. Total protein was extracted from the prefrontal cortex from the MAG1 high, MAG1 low, and control groups, as described before (54). In brief, prefrontal cortex was homogenized in radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing protease inhibitors, sonicated for 5 min at 4°C, and centrifuged for 5 min at 10,000 × *g*. Proteins were probed with primary antibodies for C1q (monoclonal, item ab71089, 1:1,000; Abcam), C1r (polyclonal, item ab205546, 1:2,000; Abcam), C3 (monoclonal, item ab225539, 1:1,000; Abcam), and C4 (polyclonal, item ab11863, 1:100; Abcam). Bands were visualized using enhanced chemiluminescence (ECL Prime Western blotting detection reagent; GE Healthcare Life Sciences). Protein values were normalized for corresponding values of β-actin. Relative optical densities were assessed using Scanalytics image analysis software (Bio-Rad).

Quantitative PCR. RNA was isolated from mouse prefrontal cortex from the MAG1 high, MAG1 low, and control groups, as described before (55). Quantitative PCR was performed according to the manufacturer's protocol using inventoried TaqMan mRNA assays (Life Technologies), as previously described (56). Fold changes between groups were evaluated using relative quantification ($\Delta\Delta C_T$ method, where C_T is threshold cycle); β-actin was the endogenous control. We examined the mRNA expression of four complement proteins, including C1q (C1qa; TaqMan assay identifier Mm00432142_m1), C1r (Mm00517767_m1), C3 (Mm01232779_m1), and C4 (C4b; Mm00437893_g1). Quantitative PCR analyses were repeated at least three times to confirm expression levels, and only results that were consistent across all three analyses were considered valid.

Statistical analyses. Data are presented as means ± standard errors of the means (SEM). Analysis of variance (ANOVA) was performed to compare means between the three MAG1-designated groups by quantitative PCR and immunoblotting experiments. The Bonferroni multiple-comparison test was applied. Statistical analyses were conducted in Graph-Pad Prism V7.03. Significance was denoted as a P of <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00139-19.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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REFERENCES

1. Gendelman HE. 2002. Neural immunity: friend or foe? J Neurovirol 8:474–479. https://doi.org/10.1080/13550280290168631.
2. Streit WJ, Mrak RE, Griffin WS. 2004. Microglia and neuroinflammation: a pathological perspective. J Neuroinflammation 1:14. https://doi.org/10.1186/1742-2094-1-14.
3. Stephan AH, Barres BA, Stevens B. 2012. The complement system: an unexpected role in synaptic pruning during development and disease. Annu Rev Neurosci 35:369–389. https://doi.org/10.1146/annurev-neuro-061010-113810.
4. Cunningham C. 2013. Microglia and neurodegeneration: the role of systemic inflammation. Glia 61:71–90. https://doi.org/10.1002/glia.22350.
5. Amor S, Puentes F, Baker D, van der Valk P. 2010. Inflammation in neurodegenerative diseases. Immunology 129:154–169. https://doi.org/10.1111/j.1365-2567.2009.03225.x.
6. Alexander JJ, Anderson AJ, Barnum SR, Stevens B, Tenner AJ. 2008. The complement cascade: Yin-Yang in neuroinflammation—neuroprotection and -degeneration. J Neurochem 107:1169–1187. https://doi.org/10.1111/j.1471-4159.2008.05668.x.
7. Dubey JP, Lindsay DS, Speer CA. 1998. Structures of Toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clin Microbiol Rev 11:267–299. https://doi.org/10.1128/CMR.11.2.267.
8. Torrey EF, Bartko JJ, Yolken RH. 2012. Toxoplasma gondii and other risk factors for schizophrenia: an update. Schizophr Bull 38:642–647. https://doi.org/10.1093/schbul/sbs043.
9. Kusbeci OY, Miman O, Yaman M, Aktepe OC, Yazar S. 2011. Could Toxoplasma gondii have any role in Alzheimer disease? Alzheimer Dis Assoc Disord 25:1–3. https://doi.org/10.1097/WAD.0b013e3181f73bc2.
10. Miman O, Kusbeci OY, Aktepe OC, Cetinkaya Z. 2010. The probable relation between Toxoplasma gondii and Parkinson's disease. Neurosci Lett 475:129–131. https://doi.org/10.1016/j.neulet.2010.03.057.
11. Nimgaonkar VL, Yolken RH, Wang T, Chang CC, McClain L, McDade E, Snitz BE, Ganguli M. 2016. Temporal cognitive decline associated with exposure to infectious agents in a population-based, aging cohort. Alzheimer Dis Assoc Disord 30:216–222. https://doi.org/10.1097/WAD.0000000000000133.
12. Kaushik M, Lamberton PH, Webster JP. 2012. The role of parasites and pathogens in influencing generalised anxiety and predation-related fear in the mammalian central nervous system. Horm Behav 62:191–201. https://doi.org/10.1016/j.yhbeh.2012.04.002.
13. Parlog A, Harsan L-A, Zagrebelsky M, Weller M, von Elverfeldt D, Mawrin C, Korte M, Dunay IR. 2014. Chronic murine toxoplasmosis is defined by subtle changes in neuronal connectivity. Dis Model Mech 7:459–469. https://doi.org/10.1242/dmm.014183.
14. David CN, Frias ES, Szu JI, Vieira PA, Hubbard JA, Lovelace J, Michael M, Worth D, McGovern KE, Ethell IM, Stanley BG, Korzus E, Fiacco TA, Binder DK, Wilson EH. 2016. GLT-1-dependent disruption of CNS glutamate homeostasis and neuronal function by the protozoan parasite Toxoplasma gondii. PLoS Pathog 12:e1005643. https://doi.org/10.1371/journal.ppat.1005643.
15. Li Y, Viscidi RP, Kannan G, McFarland R, Pletnikov MV, Severance EG, Yolken RH, Xiao J. 2018. Chronic Toxoplasma gondii infection induces anti-N-methyl-D-aspartate receptor autoantibodies and associated behavioral changes and neuropathology. Infect Immun 86:e00398-18. https://doi.org/10.1128/IAI.00398-18.
16. Pfefferkorn ER. 1984. Interferon gamma blocks the growth of Toxoplasma gondii in human fibroblasts by inducing the host cells to degrade tryptophan. Proc Natl Acad Sci U S A 81:908–912. https://doi.org/10.1073/pnas.81.3.908.
17. Horacek J, Flegr J, Tintera J, Verebova K, Spaniel F, Novak T, Brunovsky M, Bubenikova-Valesova V, Holub D, Palenicek T, Höschl C. 2012. Latent toxoplasmosis reduces gray matter density in schizophrenia but not in controls: voxel-based-morphometry (VBM) study. World J Biol Psychiatry 13:501–509. https://doi.org/10.3109/15622975.2011.573809.
18. Kempuraj D, Thangavel R, Natteru PA, Selvakumar GP, Saeed D, Zahoor H, Zaheer S, Iyer SS, Zaheer A. 2016. Neuroinflammation induces neurodegeneration. J Neurol Neurosurg Spine 1:1003.
19. Melzer TC, Cranston HJ, Weiss LM, Halonen SK. 2010. Host cell preference of Toxoplasma gondii cysts in murine brain: a confocal study. J Neuroparasitology 1:1. https://doi.org/10.4303/jnp/N100505.
20. Ferguson DJ, Hutchison WM. 1987. An ultrastructural study of the early development and tissue cyst formation of Toxoplasma gondii in the brains of mice. Parasitol Res 73:483–491. https://doi.org/10.1007/BF00535321.
21. Suzuki Y, Conley FK, Remington JS. 1989. Importance of endogenous IFN-gamma for prevention of toxoplasmic encephalitis in mice. J Immunol 143:2045–2050.
22. Hermes G, Ajioka JW, Kelly KA, Mui E, Roberts F, Kasza K, Mayr T, Kirisits MJ, Wollmann R, Ferguson DJ, Roberts CW, Hwang JH, Trendler T, Kennan RP, Suzuki Y, Reardon C, Hickey WF, Chen L, McLeod R. 2008. Neurological and behavioral abnormalities, ventricular dilatation, altered cellular functions, inflammation, and neuronal injury in brains of mice due to common, persistent, parasitic infection. J Neuroinflammation 5:48. https://doi.org/10.1186/1742-2094-5-48.
23. Evans AK, Strassmann PS, Lee IP, Sapolsky RM. 2014. Patterns of Toxoplasma gondii cyst distribution in the forebrain associate with individual variation in predator odor avoidance and anxiety-related behavior in male Long-Evans rats. Brain Behav Immun 37:122–133. https://doi.org/10.1016/j.bbi.2013.11.012.
24. Xiao J, Li Y, Gressitt KL, He H, Kannan G, Schultz TL, Svezhova N, Carruthers VB, Pletnikov MV, Yolken RH, Severance EG. 2016. Cerebral

complement C1q activation in chronic Toxoplasma infection. Brain Behav Immun 58:52–56. https://doi.org/10.1016/j.bbi.2016.04.009.
25. Schmued LC, Albertson C, Slikker W, Jr. 1997. Fluoro-Jade: a novel fluorochrome for the sensitive and reliable histochemical localization of neuronal degeneration. Brain Res 751:37–46. https://doi.org/10.1016/S0006-8993(96)01387-X.
26. Harrison JK, Jiang Y, Chen S, Xia Y, Maciejewski D, McNamara RK, Streit WJ, Salafranca MN, Adhikari S, Thompson DA, Botti P, Bacon KB, Feng L. 1998. Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. Proc Natl Acad Sci U S A 95:10896–10901. https://doi.org/10.1073/pnas.95.18.10896.
27. Xiao J, Li Y, Prandovszky E, Kannan G, Viscidi RP, Pletnikov MV, Yolken RH. 2016. Behavioral abnormalities in a mouse model of chronic toxoplasmosis are associated with MAG1 antibody levels and cyst burden. PLoS Negl Trop Dis 10:e0004674. https://doi.org/10.1371/journal.pntd.0004674.
28. O'Callaghan C, Bertoux M, Hornberger M. 2014. Beyond and below the cortex: the contribution of striatal dysfunction to cognition and behaviour in neurodegeneration. J Neurol Neurosurg Psychiatry 85:371–378. https://doi.org/10.1136/jnnp-2012-304558.
29. Ross CA, Poirier MA. 2004. Protein aggregation and neurodegenerative disease. Nat Med 10:S10–S17. https://doi.org/10.1038/nm1066.
30. Fong AY, Stornetta RL, Foley CM, Potts JT. 2005. Immunohistochemical localization of GAD67-expressing neurons and processes in the rat brainstem: subregional distribution in the nucleus tractus solitarius. J Comp Neurol 493:274–290. https://doi.org/10.1002/cne.20758.
31. Lin LH, Talman WT. 2000. *N*-Methyl-D-aspartate receptors on neurons that synthesize nitric oxide in rat nucleus tractus solitarii. Neuroscience 100:581–588. https://doi.org/10.1016/S0306-4522(00)00314-6.
32. Stevens B, Allen NJ, Vazquez LE, Howell GR, Christopherson KS, Nouri N, Micheva KD, Mehalow AK, Huberman AD, Stafford B, Sher A, Litke AM, Lambris JD, Smith SJ, John SW, Barres BA. 2007. The classical complement cascade mediates CNS synapse elimination. Cell 131:1164–1178. https://doi.org/10.1016/j.cell.2007.10.036.
33. Xiao J, Prandovszky E, Kannan G, Pletnikov MV, Dickerson F, Severance EG, Yolken RH. 2018. Toxoplasma gondii: biological parameters of the connection to schizophrenia. Schizophr Bull 44:983–992. https://doi.org/10.1093/schbul/sby082.
34. Rodgers J, Bradley B, Kennedy P. 2017. Delineating neuroinflammation, parasite CNS invasion, and blood-brain barrier dysfunction in an experimental murine model of human African trypanosomiasis. Methods 127:79–87. https://doi.org/10.1016/j.ymeth.2017.06.015.
35. Mahami-Oskouei M, Hamidi F, Talebi M, Farhoudi M, Taheraghdam AA, Kazemi T, Sadeghi-Bazargani H, Fallah E. 2016. Toxoplasmosis and Alzheimer: can Toxoplasma gondii really be introduced as a risk factor in etiology of Alzheimer? Parasitol Res 115:3169–3174. https://doi.org/10.1007/s00436-016-5075-5.
36. Perry CE, Gale SD, Erickson L, Wilson E, Nielsen B, Kauwe J, Hedges DW. 2016. Seroprevalence and seriointensity of latent Toxoplasma gondii in a sample of elderly adults with and without Alzheimer disease. Alzheimer Dis Assoc Disord 30:123–126. https://doi.org/10.1097/WAD.0000000000000108.
37. Wen X, Kudo T, Payne L, Wang X, Rodgers L, Suzuki Y. 2010. Predominant interferon-gamma-mediated expression of CXCL9, CXCL10, and CCL5 proteins in the brain during chronic infection with Toxoplasma gondii in BALB/c mice resistant to development of toxoplasmic encephalitis. J Interferon Cytokine Res 30:653–660. https://doi.org/10.1089/jir.2009.0119.
38. Frank-Cannon TC, Alto LT, McAlpine FE, Tansey MG. 2009. Does neuroinflammation fan the flame in neurodegenerative diseases? Mol Neurodegener 4:47. https://doi.org/10.1186/1750-1326-4-47.
39. Braver TS, Barch DM, Gray JR, Molfese DL, Snyder A. 2001. Anterior cingulate cortex and response conflict: effects of frequency, inhibition and errors. Cereb Cortex 11:825–836. https://doi.org/10.1093/cercor/11.9.825.
40. Khan S, Michmizos K, Tommerdahl M, Ganesan S, Kitzbichler MG, Zetino M, Garel KL, Herbert MR, Hamalainen MS, Kenet T. 2015. Somatosensory cortex functional connectivity abnormalities in autism show opposite trends, depending on direction and spatial scale. Brain 138:1394–1409. https://doi.org/10.1093/brain/awv043.
41. Levenson RW, Sturm VE, Haase CM. 2014. Emotional and behavioral symptoms in neurodegenerative disease: a model for studying the neural bases of psychopathology. Annu Rev Clin Psychol 10:581–606. https://doi.org/10.1146/annurev-clinpsy-032813-153653.
42. Brooks JM, Carrillo GL, Su J, Lindsay DS, Fox MA, Blader IJ. 2015. Toxoplasma gondii infections alter GABAergic synapses and signaling in the central nervous system. mBio 6:e01428-15. https://doi.org/10.1128/mBio.01428-15.
43. Bowie D. 2008. Ionotropic glutamate receptors & CNS disorders. CNS Neurol Disord Drug Targets 7:129–143. https://doi.org/10.2174/187152708784083821.
44. Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, Barres BA. 2012. Genomic analysis of reactive astrogliosis. J Neurosci 32:6391–6410. https://doi.org/10.1523/JNEUROSCI.6221-11.2012.
45. Veerhuis R, Nielsen HM, Tenner AJ. 2011. Complement in the brain. Mol Immunol 48:1592–1603. https://doi.org/10.1016/j.molimm.2011.04.003.
46. Schafer DP, Lehrman EK, Kautzman AG, Koyama R, Mardinly AR, Yamasaki R, Ransohoff RM, Greenberg ME, Barres BA, Stevens B. 2012. Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron 74:691–705. https://doi.org/10.1016/j.neuron.2012.03.026.
47. Aguzzi A, Barres BA, Bennett ML. 2013. Microglia: scapegoat, saboteur, or something else? Science 339:156–161. https://doi.org/10.1126/science.1227901.
48. Davis CN, Harrison JK. 2005. Proline 326 in the C terminus of murine CX3CR1 prevents G-protein and phosphatidylinositol 3-kinase-dependent stimulation of Akt and extracellular signal-regulated kinase in Chinese hamster ovary cells. J Pharmacol Exp Ther 316:356–363. https://doi.org/10.1124/jpet.105.093039.
49. Xiao J, Li Y, Yolken RH, Viscidi RP. 2018. PD-1 immune checkpoint blockade promotes brain leukocyte infiltration and diminishes cyst burden in a mouse model of Toxoplasma infection. J Neuroimmunol 319:55–62. https://doi.org/10.1016/j.jneuroim.2018.03.013.
50. Xiao J, Buka SL, Cannon TD, Suzuki Y, Viscidi RP, Torrey EF, Yolken RH. 2009. Serological pattern consistent with infection with type I Toxoplasma gondii in mothers and risk of psychosis among adult offspring. Microbes Infect 11:1011–1018. https://doi.org/10.1016/j.micinf.2009.07.007.
51. Groer MW, Yolken RH, Xiao JC, Beckstead JW, Fuchs D, Mohapatra SS, Seyfang A, Postolache TT. 2011. Prenatal depression and anxiety in Toxoplasma gondii-positive women. Am J Obstet Gynecol 204:433.e1–433.e7. https://doi.org/10.1016/j.ajog.2011.01.004.
52. Xiao J, Yolken RH. 2015. Strain hypothesis of Toxoplasma gondii infection on the outcome of human diseases. Acta Physiol (Oxf) 213:828–845. https://doi.org/10.1111/apha.12458.
53. Xiao J, Viscidi RP, Kannan G, Pletnikov MV, Li Y, Severance EG, Yolken RH, Delhaes L. 2013. The Toxoplasma MAG1 peptides induce sex-based humoral immune response in mice and distinguish active from chronic human infection. Microbes Infect 15:74–83. https://doi.org/10.1016/j.micinf.2012.10.016.
54. Xiao J, Li Y, Prandovszky E, Karuppagounder SS, Talbot CC, Jr, Dawson VL, Dawson TM, Yolken RH. 2014. MicroRNA-132 dysregulation in Toxoplasma gondii infection has implications for dopamine signaling pathway. Neuroscience 268:128–138. https://doi.org/10.1016/j.neuroscience.2014.03.015.
55. Xiao J, Li Y, Jones-Brando L, Yolken RH. 2013. Abnormalities of neurotransmitter and neuropeptide systems in human neuroepithelioma cells infected by three Toxoplasma strains. J Neural Transm (Vienna) 120:1631–1639. https://doi.org/10.1007/s00702-013-1064-3.
56. Xiao J, Jones-Brando L, Talbot CC, Jr, Yolken RH. 2011. Differential effects of three canonical Toxoplasma strains on gene expression in human neuroepithelial cells. Infect Immun 79:1363–1373. https://doi.org/10.1128/IAI.00947-10.